Effects of Polylinker uATGs on the Function of Grass HKT1 Transporters Expressed in Yeast Cells

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HvHKT1 mediates K^+ or Na^+ uniport in yeast cells if the expression promoter is joined directly to the *HvHKT1* cDNA, and Na^+–K^+ symport if a 59 nucleotide polylinker is inserted. Our results show that three ATG triplets in the polylinker decreased the synthesis of the transporter and that the lower amount of transporter caused the functional change. With the rice *HKT1* cDNA, the 59 nt polylinker changed the mode of Na^+ uptake from K^+-insensitive to K^+-inhibitable. These two modes of Na^+ uptake also occurred in rice plants.

Keywords: Plant — Potassium — Sodium — Transport — uORFs — Yeast.

Abbreviations: GFP, green fluorescent protein; ORF, open reading frame; uORF, upstream open reading frame.

Plant cells have a high number of transporters that frequently carry out redundant functions. Therefore, functional characterization of individual transporters by expression in heterologous systems has been a powerful tool in plant physiology research (Dreyer et al. 1999). This approach may be more reliable than gene knockout in the many cases in which the disruption of the transporter gene has pleiotropic effects (Rodríguez-Navarro and Rubio 2006). Among all heterologous systems that have been used for expressing plant transporters, yeast cells have played a central role due to the large number of molecular tools that can be applied, and because fungi and plants share some transporters and mechanisms of membrane energization (Rodríguez-Navarro 2000).

In contrast to these observations, functional expression of the barley and wheat HKT1 transporters in yeast cells may be misleading (Haro et al. 2005). Grass HKT transporters mediate the high-affinity Na^+ uptake that takes place in the roots of K^+-starved plants (Garciadeblás et al. 2003, Rodríguez-Navarro and Rubio 2006, Horie et al. 2007). However, barley and wheat HKT1 transporters are expressed as either Na^+ or K^+ uniporters or Na^+–K^+ symporters depending on the construct. It has been proposed that these functional changes might be caused by the expression of transporters with N-termini of different lengths (Haro et al. 2005). Here we show that this hypothesis is not correct and report that the cause is the amount of the transporter.

Several constructs are associated with functional change, but the problem can be reduced to two constructs in a widely used yeast expression vector, pYPGE15 (Brunelli and Pall 1993). In the first construct (named SLF), the *PGK1* promoter is linked directly to the barley *HvHKT1* cDNA and the function expressed is Na^+ or K^+ uniport. In the second construct (named LLF), a 59 nucleotide multiple polylinker is inserted between the *PGK1* promoter and the *HvHKT1* cDNA, and the function expressed is Na^+–K^+ symport (Haro et al. 2005; Fig. 1). We carried out a combination of systematic 5′ deletions and mutations of all codons that could serve as alternative sites of initiation of translation in the 5′ end of the *HvHKT1* open reading frame (ORF). By transforming these constructs in yeast cells we found that the length of the N-terminus of the HvHKT1 protein was not the cause of the dual function expression of *HvHKT1* in yeast cells (results not shown). Therefore, we turned our attention to the three ATG triplets in the polylinker. These ATGs originate three ORFs in the 5′-untranslated region [upstream ORFs (uORFs)] of the expressed mRNA (Haro et al. 2005), which could affect the rate of initiation of translation (Meijer and Thomas 2002, Wang and Rothnagel 2004). Therefore, we mutated the three ATG triplets and found that both the mutated LLF (mut-LLF) and SLF constructs of *HvHKT1* expressed Na^+ or K^+ uniport (Fig. 1).

To investigate whether the polylinker reduced the amount of protein, we fused the green fluorescent protein gene (*GFP*) ORF to the 3′ end of the *HvHKT1* cDNA, and the *lacZ* gene to the 3′ end of different fragments of the *HvHKT1* cDNA. In the first experiment, the HvHKT1–GFP protein was functional in both SLF and LLF constructs, producing the typical uniport and symport functions. Microscopic confocal images of both constructs clearly indicated that the fluorescence of

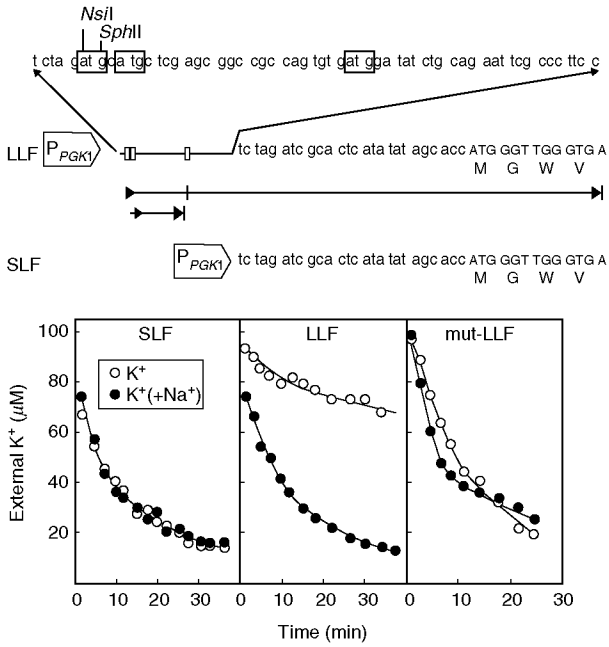
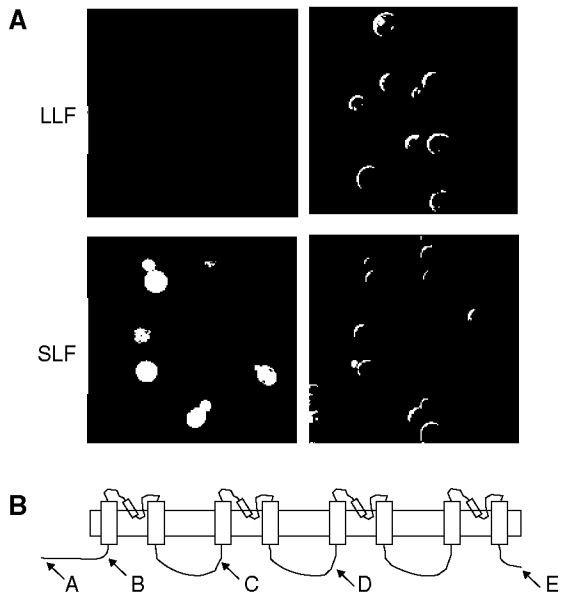


Fig. 1 Polylinker effect on the function expressed by the HvHKT1 transporter in yeast cells. The upper part of the figure shows the LLF and SLF constructs and the sequence of the 59 nt multiple cloning site. The three uATGs in the polylinker and the corresponding uORFs are indicated. The three lower panels show actual K^+ depletion data that illustrate the differences between a K^+ uniporter and an Na^+–K^+ symporter (only K^+ is shown to simplify the figure; more information can be found in Haro et al. 2005); a K^+ uniporter is not enhanced by the presence of Na^+ while in an Na^+–K^+ symporter K^+ uptake is enhanced by the presence of Na^+. The polylinker effect is eliminated by the mutation of the three uATGs (mut-LLF). The experiments were initiated by adding either K^+, at the recorded concentration, or K^+ plus 100 μM Na^+ to the suspension of transformed yeast cells. K^+ uptake by the *trk1 trk2* mutant strain transformed with an empty plasmid is undetectable in the conditions of the experiments shown.



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Construct	Fusion position	Linker	β-gal Activity (Miller units)
A	Met1	SLF	140 ± 16.5
		LLF	11 ± 4.1
B-HvHKT1	Ile46	SLF	147 ± 40.1
		LLF	10 ± 1.3
B-HvHKT1-27	Ile46	SLF	3.3 ± 0.2
		LLF	0.6 ± 0.2
E-HvHKT1	Val531	SLF	182 ± 23.2
		LLF	7 ± 2.1

Fig. 2 The polylinker decreased the synthesis of the transporter. (A) Confocal and phase contrast images of *trk1 trk2* mutant yeast cells expressing the HvHKT1–GFP fusion protein from the LLF and SLF constructs. Corresponding fluorescent pictures were taken with exactly the same settings in the microscope. (B) Schematic representations of the fusion proteins corresponding to the tested *HvHKT1Δ-lacZ* constructs; the arrows indicate the point of the fusions. (C) β-Galactosidase activity (Miller units) produced by A, B and E fusions; the results obtained with C and D fusions were not significantly different from those obtained with fusions A, B and E. In mutant 27, the first in-frame ATG triplet was mutated to CTC to prevent initiation of translation at this point. The β-galactosidase activity produced by the yeast strain transformed with a plasmid without a *lacZ* gene is undetectable in the conditions of the experiments described here.

HvHKT1–GFP protein was higher when it was produced from the SLF construct. The second set of experiments proved that the primary effect of the polylinker was to decrease protein synthesis >10-fold (Fig. 2).

Mutation of the first in-frame ATG to CTC in the *HvHKT1* ORF (*HvHKT1-27*) also changes the uniport expressed from the SLF construct into K^+–Na^+ symport, and this change involves a non-AUG initiation of translation (Haro et al. 2005). Consistent with the notion that the uniport/symport change is produced by a reduction in the amount of protein, the above-mentioned ATG/CTC mutation reduced the β-galactosidase obtained in the *lacZ* fusions about 40-fold. This result is also consistent with the notion that non-AUG initiation of translation is scant in yeast cells (Donahue and Cigan 1988). The effects of the LLF construct and ATG/CTC mutation were additive (Fig. 2).

The effect of the polylinker was exclusively on translation. By real-time PCR we found that the ratio between the *HvHKT1* and actin transcript contents in yeast cells was not affected by the polylinker (results not shown).

To investigate whether a low amount of protein was the cause of the symport function of the HvHKT1 transporter, we used the repressible promoter *MET25*. This promoter expresses approximately 10 times more

transcripts in the absence than in the presence of methionine (Thomas et al. 1989, Thomas and Surdin-Kerjan 1997). To check that the *MET25* promoter functioned correctly in our constructs, we determined the β-galactosidase activity expressed by yeast cells transformed with the SLF and LLF constructs of the P_{MET25}-*HvHKT1*Δ-*lacZ* fusion (construct B in Fig. 2). In the absence of Met, the β-galactosidase activities with the *MET25* promoter were very similar to those reported in Fig. 2C for the *PGK1* promoter in plasmid pYPGE15. In the presence of 0.5 mM methionine, the activities decreased 10- to 20-fold. We then studied the characteristics of Na^+ and K^+ uptake in yeast cells transformed with the SLF and LLF constructs of the P_{MET25}-*HvHKT1* cDNA. The presence of methionine in the growth medium favored the symport over the uniport function. This effect took place in both constructs but was more notable in that of SLF, which expressed an almost pure uniporter in the absence of methionine and close to a 50–50% uniport–symport mix in its presence (Table 1).

To investigate possible construct effects on the function of other HKT transporters in yeast cells, we expressed the rice OsHKT1 transporter from the SLF and LLF constructs. From both constructs OsHKT1 mediated only high-affinity Na^+ uptake, but that exhibited variable sensitivity to K^+ inhibition. In the SLF constructm the inhibition was low: 100 μM K^+ inhibited the initial rate of uptake from 100 μM Na^+ by 42%. An identical experiment with the LLF construct resulted in an 80% inhibition (Fig. 3). Mutation of the first in-frame ATG to CTC (*OsHKT1-27*) increased the K^+ sensitivity of the transporter expressed from both the SLF and LLF constructs with reference to the wild-type cDNA. As in β-galactosidase synthesis, the effects of the mutation and the polylinker insertion were additive and the transporter expressed by the *Oshkt1-27* cDNA from the LLF construct was extremely sensitive to K^+ inhibition (92% inhibition by 100 μM K^+ at 100 μM Na^+; Fig. 3).

Table 1 Transcript repression by the presence of methionine favored the symport function expressed from the SLF and LLF constructs of P_{MET25}-*HvHKT1* in *trk1 trk2* mutant yeast cells. Activation of Na^+ influx by K^+ and of K^+ influx by Na^+

Construct	Methionine (mM)	Na^+ ($+K^+$) (fold increase)	K^+ ($+Na^+$) (fold increase)
SLF	0	1.2 ± 0.3	1.1 ± 0.4
SLF	0.5	4.3 ± 1.5	3.3 ± 0.7
LLF	0	6.8 ± 0.8	6.32 ± 0.02
LLF	0.5	7.8 ± 1.8	8.0 ± 1.7

Activation is expressed as the ratio of the initial rate of Na^+ or K^+ uptake when the two cations were present at 100 μM divided by the rate of uptake when only one cation was present (actual time course experiments were similar to those shown in Fig. 1).

The different sensitivities of the high-affinity Na^+ uptake mediated by the SLF and LLF constructs of OsHKT1 strongly resembled the two modes of high-affinity Na^+ uptake exhibited by barley roots (Haro et al. 2005). At the beginning of K^+ starvation, high-affinity Na^+ uptake is almost insensitive to micromolar K^+ concentrations but after several hours of starvation it becomes very sensitive to K^+. In rice, only the first mode had been described (Garciadeblás et al. 2003), and we found that the second mode occurred in exactly the same way as in barley (results not shown).

In this study we have demonstrated that a reduction in the amount of synthesized transporters explains the functional change of the barley HKT1 transporter expressed in yeast cells. Similar results were observed by reducing translation, uORFs in LLF constructs, or transcription experiments with the *MET25* promoter. The electrical properties of HvHKT1 in *Xenopus* oocytes also depend on the amount of the *HvHKT1* cRNA injected (Jabnoune et al. 2007). Taken together, these results and those with OsHKT1 suggest that the functions of some HKT transporters depend on the rate of protein synthesis. The molecular basis for the functional changes of HKT transporters in yeast cells and oocytes may be linked to the physiological functions of these transporters. HKT plant transporters and TRK fungal transporters belong to the same family. ScTRK1 mediates high-affinity K^+ uptake in *Saccharomyces cerevisiae* and exhibits a dual mode of K^+ uptake too (Rodríguez-Navarro and Ramos 1984). The different functional modes of TRK and HKT transporters might depend on subtle changes in the structure of the

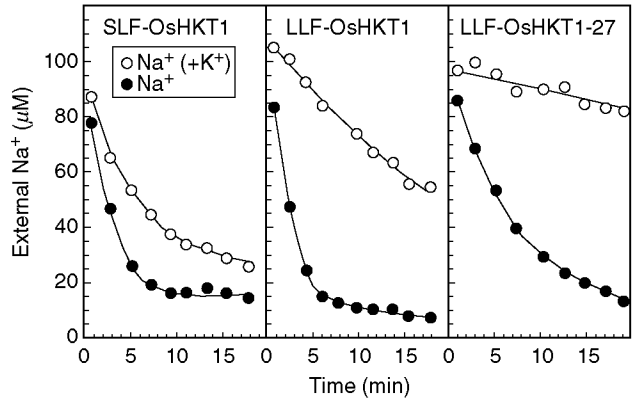

Fig. 3 The polylinker increased the sensitivity of the high-affinity K^+ uptake mediated by OsHKT1 in yeast cells. Time courses of Na^+ depletions in the absence of K^+ (filled circles) and in the presence of 100 μM K^+ (open circles). The cDNAs were cloned in either the SLF or LLF constructs (Fig. 1). The experiments were initiated by adding either Na^+, at the recorded concentration, or Na^+ plus 100 μM K^+ to the suspension of transformed yeast cells. In *OsHKT1-27* the first in-frame ATG triplet was mutated to CTC.

protein (Rodríguez-Navarro and Rubio 2006) that have not been investigated.

Finally, the experience with HKT transporters should be taken into account when expressing plant proteins in yeast cells. Although it is well known that uORFs affect the initiation of translation (Meijer and Thomas 2002, Wang and Rothnagel 2004), many polylinkers have ATG triplets. For example, *Nco*I, *Sph*I and *Nsi*I sites are included in many polylinkers although these restriction sites introduce ATG triplets.

Materials and Methods

The *S. cerevisiae* wild-type strain W303.1A (*Mat* a *ade2 ura3 leu2 his3 trp1*) was used for β-galactosidase assays, and the K^+ transport mutant strain WΔ3 (*Mat* a *ade2 ura3 trp1 trk1Δ::LEU2 trk2Δ::HIS3*; Haro et al. 1999) was used for Na^+ and K^+ transport assays.

The experiments of Na^+ and K^+ uptake in yeast cells and plant roots were carried out exactly as described previously (Haro et al. 2005).

Manipulation of nucleic acids was performed using standard protocols or, when appropriate, according to the manufacturers' instructions. The P_{MET25}-*HVHKT1* expressing gene or the P_{MET25}-*HvHKT1-lacZ* fusions described below were constructed in the YEp352 shuttle vector (Hill et al. 1986) in two steps. First, *Xba*I–*Hin*dIII fragments from the constructs in plasmid pYPGE15, which were either *HvHKT1* cDNA or derived fusions plus the *CYC1* terminator but lacking the *PGK1* promoter, were inserted into the YEp352 plasmid. Then, the *MET25* promoter included in the *Sac*I–*Xba*I fragment from plasmid pUG35 (U. Güldener and J. Hefemann http://mips.gsf.de/proj/eurofan/eurofan_2/b3/index.html) was inserted into YEp352 constructs preceding the DNA fragments inserted in the first step. All constructs and created mutations were sequenced to check that they were exactly as projected. The SLF and LLF constructs of the rice *OsHKT1* cDNA were product as described for the *HvHKT1* cDNA (Haro et al. 2005).

Four types of in-frame *HvHKT1-lacZ* fusions were constructed (Fig. 2). For the A construct in which the *lacZ* ORF substituted the complete *HvHKT1* cDNA, the *lacZ* ORF was amplified by PCR from plasmid YEp358R (Mayers et al. 1986) using the forward primer 5′-TCTAGATGTGGATC CCCGGGTACCGAGCTCGAATTC-3′, which contained an in-frame ATG, and the reverse primer 5′-CGGGTACCT TATTATTATTTTTGACACCAGACCA-3′, which contained a *Kpn*I site that was added downstream of the stop codon. The *lacZ* ORF was then inserted into the *Xba*I–*Kpn*I sites of pYPGE15. For fusion type B, in which the β-galactosidase amino acid sequence was fused following the Ile46 residue of the HvHKT1 transporter, the *lacZ* ORF was amplified using the forward primer 5′-CGACTCTAGAGGATCCCCGGG-3′, which contained a *Bam*HI site which is in the sequence of the plasmid and the reverse primer used in fusion type A. The fusion was then obtained by substituting the *Bam*HI–*Kpn*I fragment containing the *lacZ* ORF for the *Bam*HI–*Kpn*I fragment of the *HvHKT1* SLF and LLF constructs in plasmid pYPGE15. The other fusions, C (Cys178), D (Pro305) and E (Val531) (Fig. 2), were similarly constructed using convenient restriction sites. β-Galactosidase activity as expressed in Miller units was assayed in yeast cells permeabilized with chloroform and SDS as previously described (Slater and Craig 1987).

For the microscopy study, the *HvHKT1-GFP* construct was an in-frame fusion of the ORF of the *GFP* gene amplified from plasmid 35S-Adh1::GFP (Rubio-Somoza et al. 2006) to the 3′ end of the *HvHKT1* ORF. This fusion was obtained by two-step PCR. First, we amplified the *HvHKT1* (forward primer, 5′-CCTCTAGATCGCACTCATATATAGCACCA-3′; reverse primer, 5′-AGTTCTTCTCCTTTACTCATTGTTACTTTCCAG GATTTACCCATG-3′) and *GFP* (forward primer, 5′-CA TGGGTAAATCCTGGAAAGTAAGAATGAGTAAAGGAGA AGAAGT-3′; reverse primer, 5′-AGGTACCGGACGGAT CGAGCTCTTATTTGTAT-3′) ORFs, which have overlapping 3′ and 5′ ends, respectively. The second PCR was carried out using the products from the first step and the above-mentioned forward primer in the 5′ end of the *HvHKT1* ORF and reverse primer in 3′ end of the *GFP* ORF, which contained *Xba*I and *Kpn*I sites, respectively. The resulting fragment was inserted into the *Xba*I and *Kpn*I sites of plasmid pYPGE15. The GFP fluorescence signal in yeast was visualized using a Leica TCS-SP2-AOBS-UV confocal microscope (Leica Microsystems, Mannheim, Germany).

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